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(54) Title: TRANSGENIC PLANTS CONTAINING MULTIPLE DISEASE RESISTANCE GENES (57) Abstract In accordance with the present invention, there are provided transgenic plants comprising a plurality of plant-defense-associated proteins that are expressed to produce such proteins in an amount sufficient to increase the plants' resistance to plant pathogens, relative to non-transgenic plants of the same species. The transgenic plants are useful to study patterns of development, and to provide increased resistance to plant pathogens when grown in crops as a food source, and the like. Nucleic acid constructs are also provided that are useful in methods for producing the invention transgenic plants.		

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Transgenic Plants Containing Multiple
Disease Resistance Genes

The present invention relates to transgenic plants containing at least two plant-defense-associated transgenes.

BACKGROUND OF THE INVENTION

5 The interactions between plants and various soil life forms are very complex, in some instances helpful to the plant and in other instances deleterious to the plant. Fungi harmful to plants (fungal pathogens) include fungal species from a wide variety of genera,
10 including Fusarium, Pythium, Phytophthora, Verticillium, Rhizoctonia, Macrophomina, Thielaviopsis, Sclerotinia and numerous others. Plant diseases caused by fungi include pre- and post-emergence seedling damping-off, hypocotyl
15 of other symptoms. Nematodes harmful to plants (nematode pathogens) include nematode species from the genera Meloidogyne, Heterodera, Ditylenchus, and Pratylenchus. Plant diseases caused by nematodes include root galls, root rot, lesions, "stubby" root, stunting, and various
20 other rots and wilts associated with increased infection by pathogenic fungi. Some nematodes (e.g., Trichodorus, Lonoidorus, Xiphenema) can serve as vectors for virus diseases in a number of plants including Prunus, grape, tobacco and tomato.

25 Plant disease is the exception rather than the rule, as many plant pathogens express a virulent phenotype only on one or a limited number of host species. Pathogens inoculated onto a non-host species either lack the ability to grow and infect that plant, or
30 following ingress invariably encounter a successful resistance mechanism. Interactions with host species exhibit a range of specificities dependent on the

mechanism of the pathogen. One type of pathogen is the unspecialized "thug", which is necrotrophic and damages the host through production of toxins and/or enzymes. This pathogen is often equipped with inactivation or avoidance mechanisms to deal with host defenses. In contrast, "con men" pathogens grow biotrophically, avoiding serious host damage, at least in the early stages. Unlike thugs, con men pathogens do not activate host defenses nonspecifically.

For specialized pathogens having no avoidance mechanisms, host defenses are usually effective if induced. In specific interactions, following attempted infection by the pathogen, molecular signals determine whether the interaction will be incompatible or compatible. In an incompatible interaction (host resistant, pathogen avirulent), early molecular recognition is followed by rapid expression of defense responses. In a compatible interaction (host susceptible, pathogen virulent), the pathogen eludes the plant's surveillance mechanisms and disease generally ensues.

Various approaches have been utilized for attempting to control deleterious fungi and nematodes. One method is application of certain naturally occurring bacteria which inhibit or interfere with fungi or nematodes. See, for example, K.F. Baker and R.J. Cook, Biological Control of Plant Pathogens, Freeman and Co. (1974), for a description of fungi and nematodes and their interaction with plants, as well as a description of means for biological control of fungal and nematode pathogens. Another method is breeding for resistance, which is primarily focussed on the manipulation of minor resistance genes which make small quantitative contributions to the overall resistance of the plant.

Lytic enzymes have been individually transduced to form single-gene transgenic plants. For example, transfer of a tobacco basic vacuolar chitinase gene under the control of the CAMV35S promoter into the closely
5 related species, *N. sylvestris*, did not give effective protection against *C. nicotianae*, even in transgenic plants exhibiting constitutively high levels of chitinase activity (Neuhaus et al., Plant Mol. Biol., 16:141-151, 1991). See also US Patent 4,940,840 to Suslow et al.

10 Recombinant bean chitinase has been expressed in transgenic tobacco seedlings (Broglie et al., Science, 254:1194-1197, 1991). The seedlings of the chitinase-containing transgenic plants have an enhanced, but not complete, resistance to a single species of fungi. Thus,
15 it is desirable to obtain plants that have higher resistance levels to fungal pathogens than existing plants.

SUMMARY OF INVENTION

In accordance with the present invention, there
20 are provided transgenic plants. Invention plants comprise a plurality of plant-defense-associated proteins that are expressed in an amount sufficient to increase the level of the plant's resistance to plant pathogens, relative to non-transgenic plants of the same species.

25 Plants or plant cells of the present invention are useful to study patterns of development, and to provide increased resistance to plant pathogens when grown in crops as a food source, and the like. Nucleic acid constructs are provided that are useful for
30 producing the invention transgenic plants.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the results of the *Cercospora nicotinae* fungal infection assay described in the Examples for the F4 generation invention transgenic tobacco plants. O = wild type tobacco plant, □ = chitinase transgenic tobacco plant, ■ = glucanase transgenic tobacco plant, ● = chitinase/glucanase transgenic plant.

Figure 2 shows the results of the *Cercospora nicotinae* fungal infection assay described in the Examples for the heterozygous F3 generation invention transgenic tobacco plants. □ = wild type tobacco plant, ○ = chitinase transgenic tobacco plant, ● = glucanase transgenic tobacco plant, ■ = chitinase/glucanase transgenic plant.

Figures 3A (day 4 data) and 3B (day 5 data) show the results of one of the *Thanatephorus cucumeris* fungal infection assays described in the Examples. The key in descending order corresponds to each cluster of bars from left to right on the bar graph. WT= wild type tobacco plant; GLC/3 and GLC/11 = are glucanase transgenic tobacco plants; Cht/17 = chitinase transgenic tobacco plant; X/7 and X/3 are chitinase/glucanase transgenic tobacco plants.

Figures 4A, 4B, and 4C show the results of one of the *Thanatephorus cucumeris* fungal infection assays described in the Examples. The key in descending order corresponds to each cluster of bars from left to right on the bar graph. WT = wild type tobacco plant; B2 = glucanase transgenic tobacco plant; C2 = chitinase transgenic tobacco plant; and C2B2 = chitinase/glucanase transgenic tobacco plant.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided transgenic plants comprising:

- 5 a plurality of transgenes wherein each transgene encodes a plant-defense-associated protein.

In another embodiment, there are provided transgenic plants comprising:

- 10 a first transgene encoding a first overexpressed plant-defense-associated protein; and a second transgene encoding a second overexpressed plant-defense-associated protein, wherein the second plant-defense-associated protein is different from the first plant-defense-associated protein.

- 15 The term "plant" refers to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and the like. Plants contemplated for use in the practice of the present invention include both monocotyledons and dicotyledons. Exemplary monocotyledons contemplated for use in the practice of
20 the present invention include rice, wheat, maize, sorgham, barley, oat, forage grains, as well as other grains. Exemplary dicotyledons include tomato, tobacco, potato, bean, soybean, and the like.

- 25 The phrase "plants of the same species" refers to plants that have substantially completely identical genotypes, except for the inheritable nucleic acid transgenes that are introduced by the methods disclosed herein.

- 30 A "transgenic plant" refers to a plant or plant material that contains an inheritable expression cassette containing a recombinant transgene. A transgenic plant according to the present invention is a plant or plant

material that contains one or more inheritable recombinant nucleic acid expression cassettes encoding at least 2 total plant-defense-associated proteins. Preferably the invention transgenic plant contains at least 3 plant-defense-associated proteins, more preferably at least 5, with at least 10 plant-defense-associated proteins being most desirable.

The plant-defense-associated proteins may be encoded by a gene that is foreign to the recipient plant (with respect to species to which the recipient belongs, i.e., heterologous/exogenous), foreign only to the particular individual recipient (i.e., exogenous), or genetic information already possessed by the recipient (i.e., endogenous).

When an expression cassette contains an endogenous gene encoding a naturally occurring plant-defense-associated protein, the cDNA for such endogenous gene is operatively linked to a promoter different from its native promoter, such that the gene can be overexpressed relative to expression levels that naturally occur in the non-invention transgenic plant, i.e., the plant is capable of producing higher levels of the encoded protein than are naturally produced.

As used herein, an "overexpressed" plant-defense-associated protein refers to a protein that is produced in higher amounts than are produced endogenously. Overexpression can be achieved, for example, by linking a transgene to an appropriate constitutive promoter, such that the transgene is continually expressed. Alternatively, the transgene can be linked to a strong, inducible promoter so that overexpression can occur on demand.

Suitable levels of overexpression include expression of the transgene about 1.5-fold up to about 1000-fold or more over the naturally occurring level of expression of the endogenous transgene. Preferred levels of overexpression are at least about 5-fold, with at least about 10-fold over the naturally occurring level of expression of the endogenous transgene being especially preferred.

In a preferred embodiment of the invention, the transgenic plant comprises a first exogenous transgene encoding a first heterologous plant-defense-associated protein; and a second exogenous transgene encoding a second heterologous plant-defense-associated protein.

A "heterologous protein" refers to a plant-defense-associated-protein encoded by a transgene obtained from a species different from the species into which the heterologous protein is transduced (i.e., a species different from the invention transgenic plant species). Protein (or polypeptide) is a term used herein to designate a linear series of amino acid residues connected one to the other by way of a peptide bond. The heterologous protein may have distinctive properties relative to the properties of endogenously produced antimicrobial defense proteins. The heterologous protein is preferably constitutively expressed, although the natural expression of the endogenous gene encoding the protein may be inducible in response to stress.

Suitable "plant-defense-associated-proteins" contemplated for use in the invention transgenic plants are those classes of proteins that are involved in specific plant defense mechanism pathways, such as, for example, lytic enzymes, thaumatine-like proteins, α -thionin (e.g., Bohlmann et al., EMBO J., 7:1559-1565, 1988), zeamatin (e.g., Vigers et al., Mol. Plant Micro.

Interactions, 4:315-323, 1991) pathogenesis-related (PR) proteins (e.g., Bol et al., Ann. Rev. Phytopathol., 28:113-138, 1990), ribosome-inactivating-proteins (RIPs) (e.g., Leach et al., J. Biol. Chem., 266:1564-1573, 5 1990), lectins (e.g., Moreno et al., PNAS, USA, 86:7885-7889, 1989), cecropins, non-plant lysozymes, the Bacillus thuringensis toxin, enzymes involved in phytoalexin biosynthesis, proteinase inhibitors (e.g., Garcia-Olmedoz et al., Surv. Plant Mol. Cell Biol., 4:275-334, 1987), 10 inducers of plant disease resistance mechanisms, and the like.

As used herein, "lytic enzyme" refers to a protein that is able to enzymatically cleave a bond or bonds of a molecule that is essential for the survival of 15 a known pathogen. Exemplary lytic enzymes include chitinase, glucanase, cellulase, trehalase, and the like. See also, Boller T., (1987) "Hydrolytic enzymes in plant disease resistance." in T. Kosuge, E.W. Nester, eds. Plant-Microbe Interactions. Vol 2. Macmillan, New York, 20 pp 385-413, incorporated herein by reference in its entirety.

As used herein, "chitinase" refers to an enzyme that is capable of degrading chitin. In one embodiment, a chitinase construct that constitutively expresses the 25 chitinase protein is employed. Preferably, the chitinase gene employed herein is rice basic chitinase. An exemplary sequence encoding rice chitinase is set forth in SEQ ID NO:1.

Other suitable chitinase genes for use herein 30 are described, for example, in US Patent 4,940,840 to Suslow, incorporated herein by reference. See also the list of published chitinase nucleotide sequences in Table 1 of Collinge et al., Plant Journal, 3:31-40, (1993), incorporated herein by reference. In addition, suitable

chitinase genes can be obtained employing methods well-known in the art. For example, the nucleic acid molecule of SEQ ID NO:1 can be used as a probe to isolate related genes with chitinase activity from genomic or cDNA libraries of organisms known to produce chitinase proteins, such as *Trichoderma harzianum*, strain P1 (ATCC No. 74058; see also US Patent 5,173,419). See Sambrook et al., *Molecular Cloning--A Laboratory Manual*, Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y., (1989).

In another embodiment, a glucanase construct that constitutively expresses the endo-1,4- β -glucanase protein is employed. The term "glucanase" refers to an enzyme that is capable of degrading callose. Preferably, the glucanase gene employed herein is a class II alfalfa acidic β -glucanase. An exemplary sequence encoding a class II alfalfa β -glucanase is set forth in SEQ ID NO:3.

Other glucanase genes (cDNA) suitable for use herein are derived from: tomato (ATCC No. 68312; see also US Patent 5,168,064, incorporated herein by reference), avocado (Christoffersen et al., Plant Molec. Biol., 3:385, 1984) and bean (Tucker et al., Plant Physiol., 88:1257, 1988). In addition, see the tobacco glucanase sequences described in: Payne et al., Plant Mol. Biol., 15:797-808 (1990); Ward et al., Plant Physiol., 96:390-397 (1991); and the glucanase sequence described in Linthorst et al., PNAS, USA, 87:8756-8760 (1990). Suitable glucanase genes can also be obtained employing methods well-known in the art. For example, the nucleic acid molecule of SEQ ID NO:3 can be used as a probe to isolate related genes with glucanase activity (see Sambrook et al., supra).

In a presently preferred embodiment of the present invention, the first and second proteins employed in the transgenic plant are chitinase and glucanase, respectively.

The plant-defense-associated proteins described herein are encoded by recombinant transgene molecules. As used herein, the term "transgene" refers to a DNA or RNA molecule. Transgenes employed herein encode a
5 biologically active amino acid sequence (i.e., a protein). The term "plurality of transgenes" refers to greater than or equal to 2 total transgenes encoding plant-defense-associated proteins. Preferably the invention transgenic plant contains at least 3 different
10 transgenes encoding plant-defense-associated proteins, more preferably at least 5, with at least 10 different transgenes being most desirable. One of skill in the art will recognize that the transgenes employed herein encode the necessary signals required for expression of a
15 biologically active protein, such as appropriate leader peptide sequences and the like. A biologically active protein is a protein that has at least one of the physiologically properties exhibited under naturally occurring physiological conditions.

20 The transgenes encoding the plant-defense-associated proteins are typically contained in expression cassettes. The phrase "expression cassette" refers to a DNA molecule that is able to direct the transcription and translation of a structural gene (i.e., cDNA) so that a
25 desired protein is synthesized. The expression cassette comprises at least one promoter operatively linked to at least one transgene encoding a desired protein, and a transcription terminator sequence. Thus, the protein-encoding segment is transcribed under regulation of the
30 promoter region, into a transcript capable of providing, upon translation, the desired protein. Appropriate reading frame positioning and orientation of the various segments of the expression cassette are within the knowledge of persons of ordinary skill in the art;
35 further details are given in the Examples.

The promoter region refers to the portion of a gene that controls transcription of DNA to which it is operatively linked. The promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. The particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of plant-defense-associated protein to the plants resistance to microbial infection. The amount of protein needed to induce resistance may vary with the type of plant. In a preferred embodiment, the promoter employed to express the transgenes is a constitutive promoter. It should be understood that this promoter may not be the optimal one for all embodiments of the present invention.

A "constitutive" promoter is a promoter which is active under all environmental conditions and all stages of development or cell differentiation. Constitutive promoters suitable for use in the practice of the present invention are widely available and are well known in the art. Exemplary constitutive promoters include the cauliflower mosaic virus 35S ("CaMV35S") promoter (see US Patent 5,097,925, incorporated herein by reference), CaMV19S promoter, nopaline synthase (NOS), octipine synthase (OCS), the rice actin gene promoter, and the like. In addition, the DNA promoter fragments from wheat described in U.S. Patent 5,139,954; and plant promoters described in U.S. Patent 5,097,025 are suitable for use herein. A presently preferred constitutive promoter for use in the practice of the present invention is CaMV35S.

The promoters used in the DNA constructs of the present invention may be modified, if desired, to affect their control characteristics. For purposes of this description, the phrase "CaMV35S" promoter thus includes

variations of CaMV35S promoter, e.g., promoters derived by means of ligation with operator regions, random or controlled mutagenesis as well as, single, tandem or multiple copies of 35S enhancer elements, and the like.

5 In accordance with another embodiment of the present invention, there are provided nucleic acid construct(s) comprising the above-described expression cassette(s). The term "nucleic acid construct," or the abbreviated form "construct," as used herein, and
10 throughout the specification and claims, refers to a recombinant nucleic acid molecule which can include expression cassettes, origins of DNA replication, procaryotic and eucaryotic genes from various sources (such as selectable marker genes), repressor genes, as
15 well as any other sequence of nucleotides. The construct may be linear or in the circular form of a plasmid vector.

 The nucleic acid construct of the present invention, including the segments of the expression
20 cassette(s), are said to be "operably associated" with one another, such that said transgenes can be translationally expressed to produce the encoded protein under suitable conditions well-known to those of skill in the art.

25 As used herein the term "plasmid" or "vector" refers to circular, double-stranded DNA loops, which are not bound to the chromosome. One of skill in the art will recognize that the terms plasmid and vector can be used interchangeably. A plasmid contains DNA capable of
30 causing expression of DNA sequences contained therein, where such sequences are in operational association with other sequences capable of effecting their expression, such as promoter sequences, and the like. The type and number of vectors employed is not critical, so long as

greater than or equal to 2 transgenes are inheritable, e.g., capable of being expressed by each generation of plant. Suitable vectors for use in expressing the plant-defense-associated transgenes described herein include:

5 pAMVBTS (ATCC No. 53637; Barton et al., Plant Physiol., 85:1103-1109 (1987); pBI101 (Jefferson et al., EMBO J., 6:3902-3907, 1987); and the pGEM and pSP vectors (Promega, Madison, WI). Presently preferred vectors for producing invention transgenic plants are the plasmids

10 pBZ56, pM42X, and pBZ100, described hereinafter in the Examples section.

In accordance with yet another embodiment of the present invention, there are provided plant cells transformed with the above-described DNA construct(s).

15 The term "resistance," when used in the context of comparing the level of resistance between an invention transgenic plant and another plant, refers to the ability of the invention transgenic plant to maintain a desirable phenotype in the face of attack, relative to a non-

20 transgenic plant or a single-gene transgenic plant. The level of resistance can be determined by comparing the physical characteristics of the invention plant to non-transgenic plants that either have or have not been exposed to microbial infection. Exemplary physical

25 characteristics to observe include plant height, an increase in population of plants that have ability to survive microbial challenge (i.e., plants that come in contact with a compatible pathogen), delayed lesion development, reduced lesion size, and the like.

30 As used herein, "increased resistance to pathogens" refers to a level of resistance that an invention transgenic plant has to plant pathogens above a defined reference level. The defined reference level of resistance to a pathogen is the level of resistance

displayed by non-transgenic or non-invention transgenic plants of the same species. Thus, the increased resistance is measured relative to previously existing plants of the same species. In one embodiment of the invention, the resistance is substantially increased above the defined reference level, e.g., greater than or equal to 20% above, preferably 50% above, more preferably 75% above; with up to 100% above being especially preferred.

10 The phrase "non-transgenic plants of the same species" means plants of the same species that do not contain any heterologous transgenes. The respective levels of pathogen resistance can be determined using well known methods including the Fungal Infection assays described hereinafter in the Examples section.

 The phrase "transgenic plants of the same species that only express one exogenous transgene" refers to transgenic plants of the same species that only contain one transgene. These single-gene transgenic plants can contain any transgene including any one of the plurality of plant-defense-associated transgenes employed in the invention transgenic plant. Exemplary transgenic plants containing a single heterologous transgene include: glyphosate-resistant plants described in U.S. Patent 5,188,642; and the plants described in Broglie et al., Science, 254:1194-1197 (1991); and Carmora et al., Plant J., 3:457-462 (1992).

 In one embodiment of the present invention, the level of resistance imparted by the invention construct is "synergistic." Synergistic resistance refers to a level of resistance provided by at least two plant-defense-associated proteins in a single transgenic plant that is greater than the combined resistance observed for

each protein contained individually in at least two single-gene transgenic plants.

Methods of introducing the constructs employed herein into suitable host cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. According to the invention, the vector is introduced into the host cell by any suitable means, e.g., transformation employing plasmids, viral, or bacterial phage vectors, transfection, electroporation, lipofection, and the like. The heterologous DNA can optionally include sequences which allow for the extrachromosomal maintenance of the expression cassette, or said expression cassette construct can be caused to integrate into the genome of the host (as an alternative means to ensure stable maintenance in the host). See, for example: the agrobacterium mediated transformation of germinating plant seeds described in U.S. Patent 5,169,770; the plant potyvirus expression vector described in U.S. Patent 5,162,601; US Patent 5,168,064, each of which are expressly incorporated herein by reference.

In addition, cauliflower mosaic virus (CaMV) may be used as a vector for introducing nucleic acid constructs into plant cells. (Hohn et al., *"Molecular Biology of Plant Tumors,"* Academic Press, New York, pp. 549-560 (1982); Howell, U.S. Pat. No. 4,407,956). In accordance with the described method, the entire CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid is further modified by introduction of the desired sequence into unique restriction sites in the viral portion of the plasmid. The modified viral portion of the recombinant plasmid is then excised from the

parent bacterial plasmid, and used to inoculate the plant cells or plants.

Another suitable method of introducing DNA into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* that has previously been transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. *Agrobacterium* is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by *Agrobacterium* and is stably integrated into the plant genome. J. Schell, *Science*, 237:1176-1183 (1987).

Ti and Ri plasmids contain two regions essential for the production of transformed cells. One of these, named transferred DNA (T-DNA), is transferred to plant nuclei and induces tumor or root formation. The other, termed the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. The T-DNA will be transferred into a plant cell even if the vir region is on a different plasmid.

Hoekema, et al., *Nature*, 303:179-189 (1983). The transferred DNA region can be increased in size by the insertion of heterologous DNA without affecting its ability to be transferred. A modified Ti or Ri plasmid, in which the disease-causing genes have been deleted, can be used as a vector for the transfer of the gene constructs of this invention into an appropriate plant cell.

Construction of recombinant Ti and Ri plasmids in general follows methods typically used with the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include but are not limited to "shuttle vector," [Ruvkun and Ausubel, *Nature*, 298:85-88 (1981)], promoters, [Lawton et al., *Plant Mol. Biol.*, 9:315-324 (1987)] and structural genes for antibiotic resistance as a selection factor [Fraley et al., *Proc. Nat. Acad. Sci.*, 80:4803-4807 (1983)].

There are two common ways to transform plant cells with *Agrobacterium*:

(1) co-cultivation of *Agrobacterium* with cultured isolated protoplasts (which requires an established culture system that allows for culturing protoplasts and subsequent plant regeneration from cultured protoplasts), or

(2) transformation of intact cells or tissues with *Agrobacterium* (which requires that the intact plant tissues, such as cotyledons, can be transformed by *Agrobacterium*, and that the transformed cells or tissues can be induced to regenerate into whole plants.

Most dicot species can be transformed by Agrobacterium. All species which are a natural plant host for Agrobacterium are transformable in vitro. Monocotyledonous plants, and in particular, cereals, are not natural hosts to Agrobacterium. Attempts to transform them using Agrobacterium have been unsuccessful until recently. Hooykas-Van Slogteren et al., *Nature*, 311:763-764 (1984). There is growing evidence now that certain monocots can be transformed by Agrobacterium. Indeed, cereal species such as rye (de la Pena et al., *Nature*, 325:274-275 (1987), corn (Rhodes et al., *Science* 240:204-207 (1988), and rice (Shimamoto et al., *Nature*, 338:274-276 (1989) may now be transformed.

In one embodiment of the present invention, two expression cassettes containing transgenes encoding chitinase and glucanase are prepared as described above. The expression are combined into a single expression vector, to form a DNA construct which comprises two individual genes encoding plant-defense-associated proteins. The vector is then inserted into cultured *A. tumefaciens* cells which contain a disarmed Ti plasmid. In another embodiment of the present invention, two vectors are employed containing one expression cassette each. The two vectors are either transformed into a single plant or into separate plants.

Two separate transgenic plants that each contain expression cassettes having at least one transgene can be sexually crossed using well-known methods to produce a transgenic plant of the present invention. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of

Agrobacterium, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, and transformation using viruses. For example, the construct described above can be microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA. Crossway, *Mol. Gen. Genetics*, 202:179-185 (1985). The genetic material may also be transferred into the plant cell using polyethylene glycol, Krens, et al., *Nature*, 296:72-74 (1982).

Another method of introduction of transgene segments is high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface (see, for example, Klein, et al., *Nature*, 327:70-73, 1987). Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies (see, for example, Fraley, et al., *Proc. Natl. Acad. Sci. USA*, 79:1859-1863, 1982).

The DNA may also be introduced into the plant cells by electroporation. Fromm et al., *Proc. Natl. Acad. Sci. USA*, 82:5824 (1985). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

After transformation, transformed plant cells or plants comprising the invention nucleic acid constructs can be identified employing well-known

methods. For example, a selectable marker, such as those discussed above, is typically used. Transformed plant cells can be selected by growing the cells on growth medium containing an appropriate antibiotic. The
5 presence of opines can also be used if the plants are transformed with *Agrobacterium*.

After selecting the transformed cells, one can confirm expression of the desired heterologous gene. Simple detection of mRNA encoded by the inserted DNA can
10 be achieved by well known methods in the art, such as Northern blot hybridization. The inserted sequence can be identified by Southern blot hybridization, as well.

Once the presence of the desired transgenes is confirmed, whole plant regeneration is desired. All
15 plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention. Some suitable plants include, for example, species from the genera
20 *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*,
25 *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hererocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*, *Malus*, *Apium*, *Datura*, and the like.

Plant regeneration from cultured protoplasts is
30 described in Evans et al., *Handbook of Plant Cell Cultures*, Vol. 1: (MacMillan Publishing Co. New York, 1983); and Vasil, I.R. (ed.), *Cell Culture and Somatic Cell Genetics of Plants*, Acad. Press, Orlando, Vol. I, 1984, and Vol. III, 1986.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beet, cotton, fruit trees, and legumes.

5 Means for regeneration vary from species to species, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently
10 rooted. Alternatively, embryo formation can be induced employing well-known plant hormones in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins.
15 It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then
20 regeneration is usually reproducible and repeatable.

With the methods of the present invention, one can generate a transgenic plant containing at least a plurality of plant-defense-associated proteins, an origin of replication from either yeast, insect or mammalian
25 cells, and a selectable marker gene for the expression of the plant-defense-associated proteins described herein.

The invention will now be described in greater detail by reference to the following non-limiting examples.

30

EXAMPLES

The nomenclature used hereafter and the laboratory procedures in recombinant DNA technology

described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook et al., *Molecular Cloning--A Laboratory Manual*, Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y., (1989). The manual is hereinafter referred to as "Sambrook." Other general references are provided throughout this document. The procedures therein are well known in the art and are described herein for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Vector Preparation

Construction of plasmid pBZ56

A SphI fragment of rice chitinase RCH10 gene (SEQ ID NO: 1) from plasmid pRCH10 (described in Zhu et al., *Plant J.*, 3:203-212, 1993) was subcloned into pSP72 (Promega, Madison, WI) to give pBZ5B. A Sac2/KpnI fragment of RCH10 was subcloned into pSP72 to give pBZ52. The NcoI/EcoRV fragment of pBZ52 was inserted into pBZ5B NcoI/PvuII sites to give pBZ54. The EcoRV/BglIII fragment of pBZ54 was inserted into pGEM721 BamHI/EcoRV sites to give pBZ55 [pGEM721 is a pGEM7 (Promega, Madison, WI) plasmid containing CaMV 35S promoter]. The HpaI/SacI fragment of pBZ55 was inserted into pBI101.1 (Jefferson et al., *EMBO J.*, 6:3902-3907, 1987) to give pBZ56.

Construction of pM42X

A lambda ZAPII (Stratagene, La Jolla, CA) cDNA library prepared from poly (A) RNA isolated from alfalfa suspension cells 2, 3, and 4 h after elicitation with a fungal cell wall preparation (Dalkin et al., Physiol. and Mol. Plant Path., 37:293-307, 1990) was screened using a bean glucanase cDNA as probe (Edington et al., Plant Mol. Biol., 16:81-94, 1991). Positive plaques identified on duplicate filters were purified through two subsequent rounds of screening. Plasmids were then rescued in pBluescript SK- by use of the helper phage R408 (Stratagene), and insert size and diversity determined by restriction mapping. The largest insert that hybridized to the bean glucanase probe was subcloned into pGEM-3Zf(+) (Promega) to yield the plasmid "pAglu1", which contains the entire coding region for an isoform of acidic alfalfa β -1,3-glucanase (SEQ ID NO: 3).

A BamHI/XhoI fragment of pAglu1 containing an alfalfa β -glucanase gene was inserted into pMON530 (Monsanto, St. Louis, MO) BglIII/XhoI sites to give pM4. The BamHI complete/PstI partial digested fragment of pM4 was inserted into pSP72 (Promega) to give pM42. The PvuII/SacI fragment of pM42 fragment was inserted into the SmaI/SacI site of pBI121 (Jefferson et al., EMBO J., 6:3902-3907, 1987) to give pM42X.

Construction of pBZ100

The plasmid pBZ55, described above, was digested with EcoRV/SalI, filled in with DNA polymerase Klenow fragment and religated to give pBZ55M. An HpaI/XhoI fragment of pBZ55M was inserted into pGEM7 (Promega) to give pBZ55M-7. A SacI fragment of pBZ55M-7 was inserted into pM42X to give pBZ100.

Tobacco Transformation

Plasmids pBZ56 and pM42X were directly transformed in *Agrobacterium tumefaciens* LBA4404, and independent transgenic tobacco plants containing either a rice chitinase or alfalfa glucanase transgene were generated by the leaf disc method (see, e.g., Rogers et al., Meth. in Enzymol., 118:627-640, 1986). Transformed plants were selected on Murashige and Skoog medium (see Murashige and Skoog, Physiol. Plant, 15:673, 1962) containing 200 µg/ml kanamycin and 500 µg/ml carbenicillin, and grown at 25° C under a 16 hour light (115mE)/8 hour dark cycle for several weeks. The seedlings from the transformed plants were then moved into soil.

Approximately 24 pBZ56 and 20 pM42X transgenic plants were confirmed as transformants by Southern blot hybridization. Expression of rice chitinase and alfalfa-glucanase transgenes was checked by conventional Northern blot and Western blot analysis. Several F2 generation transgenic lines that expressed high levels of either one of the above transgenes were selected for mating to produce an invention transgenic plant.

Preparation of transgenic tobacco plant containing multiple plant-defense-associated genes

The F2 high level expression homozygous transgenic lines of pBZ56 and pM42X transformants were crossed with each other using routine methods. Polymerase chain reaction (PCR) analysis was employed to confirm that the heterozygous F3 generation (i.e., crossed plants) contained both transgenes. After confirmation, the F3 generation seeds were harvested and F4 generation plants were produced. F4 generation plants that were homozygous for both the chitinase and glucanase

transgenes were selected and their genotypes were confirmed by back-cross and PCR analysis. The F4 plants were used for fungal infection assays.

5 Preparation of transgenic rice plant containing multiple
plant-defense-associated genes

10 The plasmid pBZ100 and a plasmid containing a hygromycin-resistant gene were introduced into rice embryos employing a commercially available ballistic micro-projectile device. The bombarded embryos were grown on N6 medium (see Chu et al., Scientia Sinica, 18:659-668, 1975) containing hygromycin. Regenerated hygromycin resistant plants were analyzed by PCR and Southern blot analysis for the presence of inheritable pBZ100 DNA. The results indicate that the genomic DNA of
15 the rice transformants contain inheritable copies of both transgenes.

Assay for resistance of Tobacco transgenic plants to
Cercospora Nicotinae Fungal Infection

20 Both F3 and F4 generation invention transgenic tobacco plants, described above, were subjected to the following fungal infection assay. The results are shown in Figures 3 and 4, respectively.

25 *Cercospora nicotianae* (available from American Type Culture Collection under ATCC Nos. 18366 and 18367) were cultured by suspending mycelial fragments in sterile ddH₂O. A tobacco leaf suspension was prepared containing 200 ml V8 juice, 3.0 g CaCO₃, 800 ml water, pH to 6.15, 18 g agar, 1 g dried tobacco leaves (dry several large leaves in the microwave for about 10 min or 'defrost';
30 grind in a mortar and pestle), and autoclaved for 20-25 minutes. Approximately 0.5-1.0 ml of the tobacco leaf suspension was spread onto a petri plate. The contents

of the plate were incubated at 25° C with continuous light for about 4-6 weeks until white-ish mycelial covered the plate evenly.

The F3 and F4 generations of an invention tobacco transgenic plant (approximately 100 of each), and appropriate controls, were grown to about 5-6 weeks old. Mycelia were cut evenly into small squares (approximately 2 mm²) with a sterile scalpel. The mycelial agar squares were adhered to the underside and between the veins of tobacco leaves (not the newest leaf: usually the 2nd or 3rd from the top) using 3/4" waterproof surgical tape (Blenderm, 3M). The plants were incubated at 20° C, for 4-10 days using a 16 hour daylength.

Symptoms of fungal infection become visible in approximately 24-96 hours. Initially, small pin-pricks are just visible, with perhaps some browning of the leaf tissue over the inoculum. This progresses to a grey-ish, dry lesion of increasing diameter. The relative level of fungal infection was scored using a numerical rating system as follows:

- 0 - no symptoms, maybe a little browning;
- 1 - 'pin-pricks,' but no contiguous, measurable lesion;
- >1 - (measuring the lesions, taking the widest portion as one measurement, then the perpendicular as the other; multiply these to get an approximate disease area in mm².)

Figure 1 shows the results of the fungal infection assay for the F4 generation invention transgenic tobacco plants. The results indicate that the lesion sizes for the invention transgenic plant were at all times less than the lesion sizes observed for the controls, e.g., the wild type tobacco plant and the single-gene transgenic tobacco plants containing only one

of either the chitinase or β -glucanase genes. Thus, the invention transgenic plant has a higher level of resistance to lesions caused by fungal pathogens than each of the control tobacco plants.

5 Figure 2 shows the results of the fungal infection assay for the heterozygous F3 generation invention transgenic tobacco plants. The results indicate that the infection frequency for the invention transgenic tobacco plants was essentially less than the
10 infection frequencies observed for the controls, e.g., the wild type tobacco plant and the single-gene transgenic tobacco plants containing only one of either the chitinase or β -glucanase genes. Thus, the invention transgenic plant has a higher level of resistance to
15 fungal infection caused by fungal pathogens than each of the control tobacco plants.

Assay for resistance of Tobacco transgenic plants to
Thanatephorus cucumeris Fungal Infection

 In the imperfect stage, *T. cucumeris* from
20 anastomosis group 3 (such as the strain ATCC No. 62149) causes stem lesions ("sore shin") similar to the pathogen *Rhizoctonia solani*. However, basidiospores of the fungus form in the perfect stage. Germinating basidiospores form appressoria that penetrate tobacco leaves directly,
25 leading to the formation of lesions ('target spot'). Factors that favor development of the perfect stage are temperatures within the range of 16-28° C and leaf wetness.

T. cucumeris were cultured by inoculating
30 autoclaved rice media (50 g Uncle Ben's Converted Rice, 25 ml doubly distilled water in 250 ml flasks; cover with foil and autoclave 25 minutes, see Shew and Main, Plant Disease, 74:1009-1013, 1990) with squares of mycelia from

PDA (potato dextrose agar) plates. The plates were incubated at 22-25° C for 2-4 weeks with continuous light (the rice looks "mummified"--white and powdery).

To inoculate the invention transgenic tobacco plant, and appropriate controls, inoculum was ground in a blender until very few rice grains remained (approximately 3 X 45 seconds, scraping down the sides in between). Starting with the 0 level, inoculum was mixed into dry potting mix at the rate of 0, 1, 2 and 5g rice mixture/liter of soil and distributed to 9-well pot forms. Eighteen 4-5 week old tobacco seedlings were transplanted to the wells, being careful to avoid carry-over from high inoculum levels to low ones (preferably use the wooden end of a separate disposable cotton applicator for each level to make a hole and firm the soil around the stem). Subsequently, pot-sets were placed in a flat cover with a plastic dome and incubated at 20° C for 4-7 days using a 16 hour daylength. The dome was removed and sprinkled with water as needed to maintain moist conditions.

Symptoms of fungal infection in the highest inoculum become evident in approximately 3-4 days. Leaves touching the soil become grey-ish and wilted; upper leaves wilt; stems become girdled at the soil line, brown-ish and constricted. For the results shown in Figures 3A and B, the relative level of fungal infection was scored using a numerical rating system as follows:

- 0 - No symptoms;
- 1 - A diseased leaf or wilting, but plant may survive;
- 2 - Severely constricted stem indicating plant will die; flat-out, wilted plant.

The results of the assay at days 4 and 5 are shown in Figures 3A and 3B, respectively. Figure 3A (day

4) indicates that the invention transgenic plant designated X/3, when grown in potting soil containing 5g of the above-described fungal rice mixture/liter, has a higher level of resistance to the pathogen than the control tobacco plants which include a wild type tobacco plant and single-gene transgenic tobacco plants containing either one of the chitinase or β -glucanase gene.

Figure 3B (day 5) indicates that both the X/7 and X/3 invention transgenic tobacco plants, when grown in potting soil containing 2g and 5g of rice mixture/liter, have a higher level of resistance to the pathogen than the control tobacco plants which include a wild type tobacco plant and single-gene transgenic tobacco plants containing either one of the chitinase or β -glucanase gene.

A similar assay was conducted using F5 transgenic tobacco plants that were homozygous for both the chitinase and β -glucanase genes. In this assay 4g, 15g, and 45g of the rice mixture/liter was employed, and the plants were scored as either healthy or diseased. The results are shown in Figures 4A (4g), 4B (15g), and 4C (45g). The results indicate that the invention transgenic plant containing two plant-defense-associated proteins clearly has increased resistance to fungal pathogens relative to non-transgenic plants and transgenic plants of the same species that only express one of said plant-defense-associated proteins.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Lamb, Christopher J.
Zhu, Qun
Maher, Eileen A.
Dixon, Richard A.
- (ii) TITLE OF INVENTION: TRANSGENIC PLANTS CONTAINING MULTIPLE
DISEASE RESISTANCE GENES
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
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 - (B) STREET: 444 South Flower Street, Suite 2000
 - (C) CITY: Los Angeles
 - (D) STATE: CA
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 90071-2921
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/999,999
 - (B) FILING DATE: 15-DEC-1990
 - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Reiter, Stephen E.
 - (B) REGISTRATION NUMBER: 31,192
 - (C) REFERENCE/DOCKET NUMBER: P41 9391
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 619-546-4737
 - (B) TELEFAX: 619-546-9392

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1151 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 55..1062
 - (D) OTHER INFORMATION: /product= "RICE CHITINASE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCC AAC TGC CTC TGC TGC AGC CAG TTC GGC TGG TGC GGC TCC ACC TCC	201
Pro Asn Cys Leu Cys Cys Ser Gln Phe Gly Trp Cys Gly Ser Thr Ser	
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Asp Tyr Cys Gly Ala Gly Cys Gln Ser Gln Cys Ser Arg Leu Arg Arg	
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Arg Arg Pro Asp Ala Ser Gly Gly Gly Gly Ser Gly Val Ala Ser Ile	
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GTG TCG CGC TCG CTC TTC GAC CTG ATG CTG CTC CAC CGC AAC GAT GCG	345
Val Ser Arg Ser Leu Phe Asp Leu Met Leu Leu His Arg Asn Asp Ala	
85 90 95	
GCG TGC CCG GCC AGC AAC TTC TAC ACC TAC GAC GCC TTC GTC GCC GCC	393
Ala Cys Pro Ala Ser Asn Phe Tyr Thr Tyr Asp Ala Phe Val Ala Ala	
100 105 110	
GCC AGC GCC TTC CCG GGC TTC GCC GCC GCG GGC GAC GCC GAC ACC AAC	441
Ala Ser Ala Phe Pro Gly Phe Ala Ala Ala Gly Asp Ala Asp Thr Asn	
115 120 125	
AAG CGC GAG GTC GCC GCG TTC CTT GCG CAG ACG TCC CAC GAG ACC ACC	489
Lys Arg Glu Val Ala Ala Phe Leu Ala Gln Thr Ser His Glu Thr Thr	
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GGC GGG TGG GCG ACG GCG CCC GAC GGC CCC TAC ACG TGG GGC TAC TGC	537
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TTC AAG GAG GAG AAC GGC GGC GCC GGC CCG GAC TAC TGC CAG CAG AGC	585
Phe Lys Glu Glu Asn Gly Gly Ala Gly Pro Asp Tyr Cys Gln Gln Ser	
165 170 175	
GCG CAG TGG CCG TGC GCC GCC GGC AAG AAG TAC TAC GGC GCG GGT CCC	633
Ala Gln Trp Pro Cys Ala Ala Gly Lys Lys Tyr Tyr Gly Arg Gly Pro	
180 185 190	
ATC GAG CTC TCC TAC AAC TTC AAC TAC GGC CCG GCG GGC CAG GCC ATC	681
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GGC GCC GAC CTG CTC GGC GAC CCG GAC CTC GTG GCG TCT GAC GCC ACC	729
Gly Ala Asp Leu Leu Gly Asp Pro Asp Leu Val Ala Ser Asp Ala Thr	
210 215 220 225	
GTC TCC TTC GAC ACG GCC TTC TGG TTC TGG ATG ACG CCG CAG TCG CCC	777
Val Ser Phe Asp Thr Ala Phe Trp Phe Trp Met Thr Pro Gln Ser Pro	
230 235 240	

32

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GCC GAC CCG ATC GGC TTC TAC AAG CGC TAC TGC GAC ATC CTC GGC GTC Ala Asp Arg Ile Gly Phe Tyr Lys Arg Tyr Cys Asp Ile Leu Gly Val 290 295 300 305	969
AGC TAC GGC GCC AAC TTG GAT TGC TAC AGC CAG AGG CCT TCG GCT CCT Ser Tyr Gly Ala Asn Leu Asp Cys Tyr Ser Gln Arg Pro Ser Ala Pro 310 315 320	1017
CCT AAG CTT CGC CTA CCT AGC TTC CAC ACA GTG ATA AAT AAT CAC Pro Lys Leu Arg Leu Pro Ser Phe His Thr Val Ile Asn Asn His 325 330 335	1062
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(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 336 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Ala Ala Val His Ala Glu Gln Cys Gly Ser Gln Ala Gly Gly Ala Val 20 25 30
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Ser Asp Tyr Cys Gly Ala Gly Cys Gln Ser Gln Cys Ser Arg Leu Arg 50 55 60
Arg Arg Arg Pro Asp Ala Ser Gly Gly Gly Gly Ser Gly Val Ala Ser 65 70 75 80
Ile Val Ser Arg Ser Leu Phe Asp Leu Met Leu Leu His Arg Asn Asp 85 90 95
Ala Ala Cys Pro Ala Ser Asn Phe Tyr Thr Tyr Asp Ala Phe Val Ala 100 105 110
Ala Ala Ser Ala Phe Pro Gly Phe Ala Ala Ala Gly Asp Ala Asp Thr 115 120 125
Asn Lys Arg Glu Val Ala Ala Phe Leu Ala Gln Thr Ser His Glu Thr 130 135 140

33

Thr Gly Gly Trp Ala Thr Ala Pro Asp Gly Pro Tyr Thr Trp Gly Tyr
 145 150 155 160
 Cys Phe Lys Glu Glu Asn Gly Gly Ala Gly Pro Asp Tyr Cys Gln Gln
 165 170 175
 Ser Ala Gln Trp Pro Cys Ala Ala Gly Lys Lys Tyr Tyr Gly Arg Gly
 180 185 190
 Pro Ile Gln Leu Ser Tyr Asn Phe Asn Tyr Gly Pro Ala Gly Gln Ala
 195 200 205
 Ile Gly Ala Asp Leu Leu Gly Asp Pro Asp Leu Val Ala Ser Asp Ala
 210 215 220
 Thr Val Ser Phe Asp Thr Ala Phe Trp Phe Trp Met Thr Pro Gln Ser
 225 230 235 240
 Pro Lys Pro Ser Cys Asn Ala Val Ala Thr Gly Gln Trp Thr Pro Ser
 245 250 255
 Ala Asp Asp Gln Arg Ala Gly Arg Val Pro Gly Tyr Gly Val Ile Thr
 260 265 270
 Asn Ile Ile Asn Gly Gly Leu Glu Cys Gly His Gly Glu Asp Asp Arg
 275 280 285
 Ile Ala Asp Arg Ile Gly Phe Tyr Lys Arg Tyr Cys Asp Ile Leu Gly
 290 295 300
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1374 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 52..1158
- (D) OTHER INFORMATION: /product= "ALFALFA BETA-GLUCANASE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 CTA TTG GGA TTG TTC ACA ATA AAC CTC ATT CCC ACA GCA GAT GCT CAA 153
 Leu Leu Gly Leu Phe Thr Ile Asn Leu Ile Pro Thr Ala Asp Ala Gln
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34

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35

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Gln Lys Lys Tyr Pro Phe Gly Phe Gly Gly Glu Arg Met Gly Ile Val	
340 345 350	
AAT GGT GAC TTC AAT GCA ACT ATT TCT CTT AAG AGT GAC ATG TAAGAAAAAA	1165
Asn Gly Asp Phe Asn Ala Thr Ile Ser Leu Lys Ser Asp Met	
355 360 365	
ATCAAGGTTT TCAAGATTTG AGTGGTTTTA TGCATAAAAT AAGAGAATTT CTCGTGTATG	1225
TATTTTATTT ATCTTTCTTT TTTGGGTGT AGAAAATTTG GAATGCTTGA GTTTCCTATT	1285
TCTCTAAAAA TTAATGTCTT GTGATGAAGT TATATGAATA TTTATATAAA GCGTAACTTT	1345
CCAAATTATT TAATACTATT TTTCAAAAAA	1374

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 368 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Pro	Ser	Phe	Phe	Ala	Pro	Thr	Arg	Arg	Phe	Ser	Leu	Ala	Ser	Pro
1				5					10					15	
Leu	Leu	Leu	Leu	Gly	Leu	Phe	Thr	Ile	Asn	Leu	Ile	Pro	Thr	Ala	Asp
			20					25					30		
Ala	Gln	Ile	Gly	Val	Cys	Tyr	Gly	Met	Met	Gly	Asn	Asn	Leu	Pro	Pro
		35					40					45			
Ala	Asn	Glu	Val	Ile	Asp	Leu	Tyr	Lys	Ala	Asn	Asn	Ile	Lys	Arg	Met
	50					55					60				
Arg	Leu	Tyr	Asp	Pro	Asn	Gln	Ala	Ala	Leu	Asn	Ala	Leu	Arg	Asn	Ser
65					70				75					80	
Gly	Ile	Glu	Leu	Ile	Leu	Gly	Val	Pro	Asn	Ser	Asp	Leu	Gln	Ser	Leu
			85						90					95	
Ala	Thr	Asn	Ser	Asp	Asn	Ala	Arg	Gln	Trp	Val	Gln	Arg	Asn	Val	Leu
		100				105						110			
Asn	Phe	Trp	Pro	Ser	Val	Lys	Ile	Lys	Tyr	Ile	Ala	Val	Gly	Asn	Glu
	115					120					125				
Val	Ser	Pro	Val	Gly	Gly	Ser	Ser	Trp	Leu	Gly	Gln	Tyr	Val	Leu	Pro
	130					135					140				
Ala	Thr	Gln	Asn	Ile	Tyr	Gln	Ala	Ile	Arg	Ala	Lys	Asn	Leu	His	Asp
145					150				155					160	

36

Gln Ile Leu Val Ser Thr Ala Ile Asp Met Thr Leu Ile Gly Asn Ser
 165 170 175
 Phe Pro Pro Ser Lys Gly Ser Phe Arg Asn Asp Val Arg Ala Tyr Leu
 180 185 190
 Asp Pro Phe Ile Gly Tyr Leu Val Tyr Ala Gly Ala Pro Leu Leu Val
 195 200 205
 Asn Val Tyr Pro Tyr Phe Ser His Val Gly Asn Pro Arg Asp Ile Ser
 210 215 220
 Leu Pro Tyr Ala Leu Phe Thr Ser Pro Gly Val Met Val Gln Asp Gly
 225 230 235 240
 Pro Asn Gly Tyr Gln Asn Leu Phe Asp Ala Met Leu Asp Ser Val His
 245 250 255
 Ala Ala Leu Asp Asn Thr Gly Ile Gly Trp Val Asn Val Val Val Ser
 260 265 270
 Glu Ser Gly Trp Pro Ser Asp Gly Gly Ala Thr Ser Tyr Asp Asn Ala
 275 280 285
 Arg Ile Tyr Leu Asp Asn Leu Ile Arg Tyr Glu Gly Lys Gly Thr Pro
 290 295 300
 Arg Arg Pro Trp Ala Thr Glu Thr Tyr Ile Phe Ala Met Phe Asp Glu
 305 310 315 320
 Asn Gln Lys Ser Pro Glu Leu Glu Lys His Phe Gly Val Phe Tyr Pro
 325 330 335
 Asn Lys Gln Lys Lys Tyr Pro Phe Gly Phe Gly Gly Glu Arg Met Gly
 340 345 350
 Ile Val Asn Gly Asp Phe Asn Ala Thr Ile Ser Leu Lys Ser Asp Met
 355 360 365

That which is claimed is:

1. A transgenic plant comprising:
a plurality of transgenes, wherein each
transgene encodes a plant-defense-associated protein.
2. A plant according to Claim 1, wherein said
plant-defense-associated proteins are expressed as
biologically active proteins.
3. A plant according to Claim 1, wherein each
of said transgenes is operatively linked to a promoter
sequence, such that said plant-defense-associated
proteins are constitutively expressed.
4. A plant according to Claim 1, wherein at
least one of said transgenes is exogenous to said plant.
5. A plant according to Claim 1, wherein at
least one of said plant-defense-associated proteins is a
lytic enzyme.
6. A plant according to Claim 1, wherein both
of said plant-defense-associated proteins are lytic
enzymes.
7. A plant according to Claim 5, wherein said
lytic enzyme is chitinase.
8. A plant according to Claim 7, wherein said
chitinase is rice chitinase.
9. A plant according to Claim 6, wherein one
of said lytic enzymes is glucanase.
10. A plant according to Claim 9, wherein said
glucanase is alfalfa glucanase.

11. A plant according to Claim 1, wherein said plant has increased resistance to fungal pathogens relative to non-transgenic plants of the same species.

12. A plant according to Claim 1, wherein said plant has increased resistance to fungal pathogens relative to transgenic plants of the same species that only express one of said transgenes.

13. A plant according to Claim 12, wherein said increased resistance is synergistic.

14. A plant according to Claim 1, wherein said plant is a monocot.

15. A monocot plant according to Claim 14, wherein said plant is selected from: barley, wheat, maize, oat, rice, sorgham.

16. A monocot plant according to Claim 15, wherein said plant is a rice plant.

17. A plant according to Claim 1, wherein said plant is a dicot.

18. A dicot plant according to Claim 17, wherein said plant is selected from: tomato, tobacco, potato, bean, and soybean.

19. A dicot plant according to Claim 18, wherein said plant is a tobacco plant.

20. A transgenic plant comprising:

a first exogenous transgene encoding an overexpressed and biologically active rice chitinase protein; and

5 a second exogenous transgene encoding an overexpressed and biologically active alfalfa glucanase protein,

wherein:

10 said transgenes are constitutively expressed, and

said plant has increased resistance to fungal pathogens relative to plants that do not constitutively express both of said transgenes.

21. A plant according to Claim 20, wherein said plant is a tobacco plant.

22. A nucleic acid construct comprising:

a first transgene encoding a first plant-defense-associated protein; and

5 a second transgene encoding a second plant-defense-associated protein, and

wherein each of said transgenes are operatively linked to a promoter.

23. A construct according to Claim 22, wherein said promoter is a constitutive promoter.

24. A construct according to Claim 22, wherein said first plant-defense-associated protein is a chitinase protein.

25. A construct according to Claim 24, wherein said chitinase protein is rice chitinase.

26. A construct according to Claim 22, wherein said second plant-defense-associated protein is a glucanase protein.

27. A construct according to Claim 26, wherein said glucanase protein is alfalfa glucanase.

28. A construct according to Claim 22, wherein said construct imparts to transgenic plants containing same, increased resistance to fungal pathogens relative to non-transgenic plants of the same species that do not
5 contain said construct.

29. A vector comprising the construct of Claim 22.

30. A plant cell containing the vector of Claim 29.

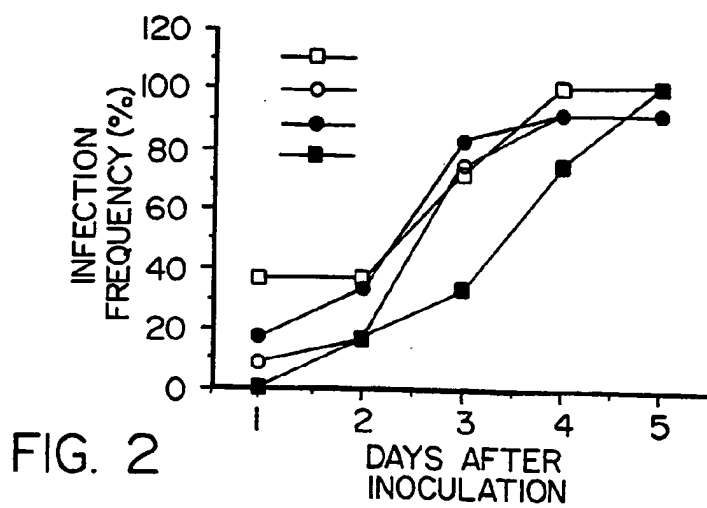
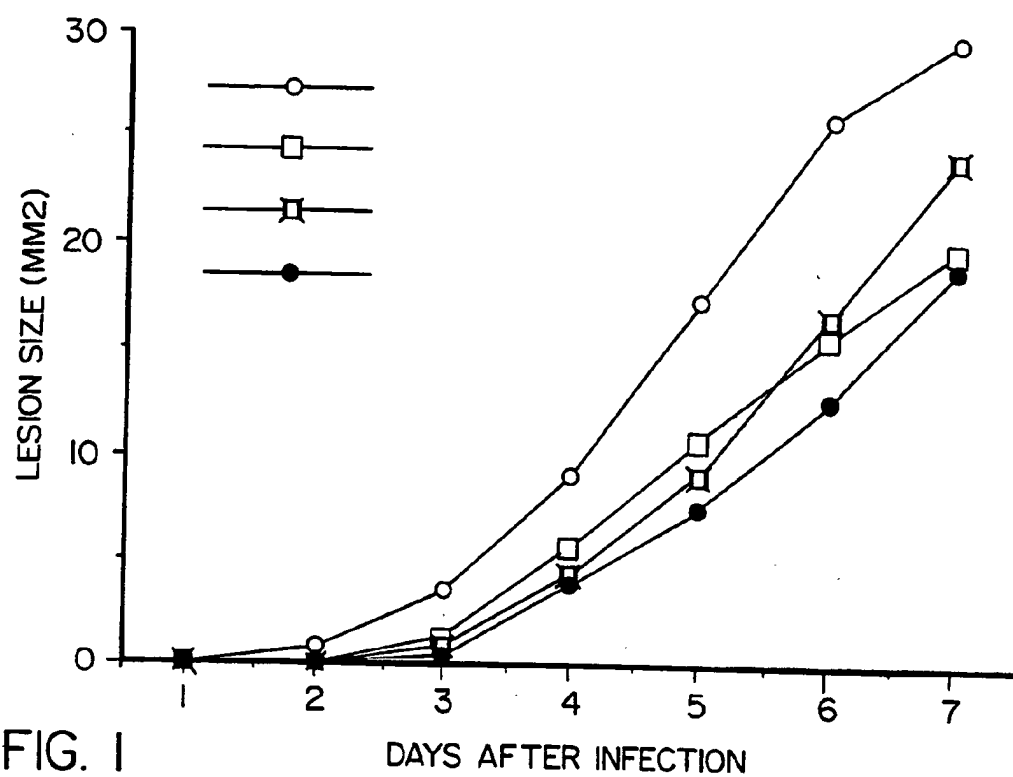
31. A method to increase disease resistance of a plant, said method comprising:

introducing the nucleic acid
construct of Claim 22 into the genome of said
plant.

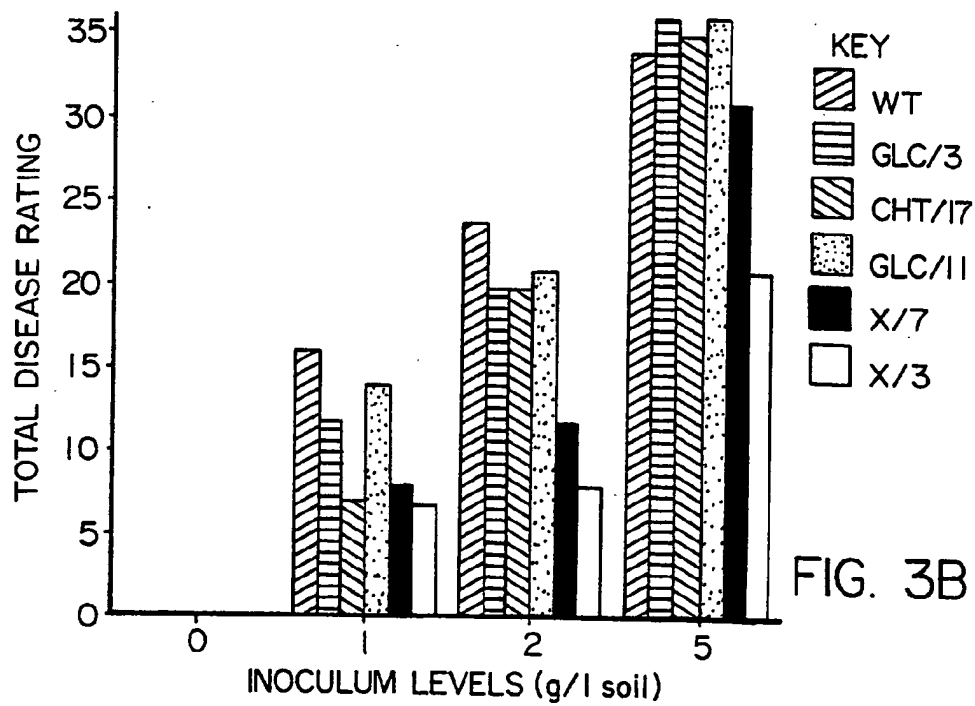
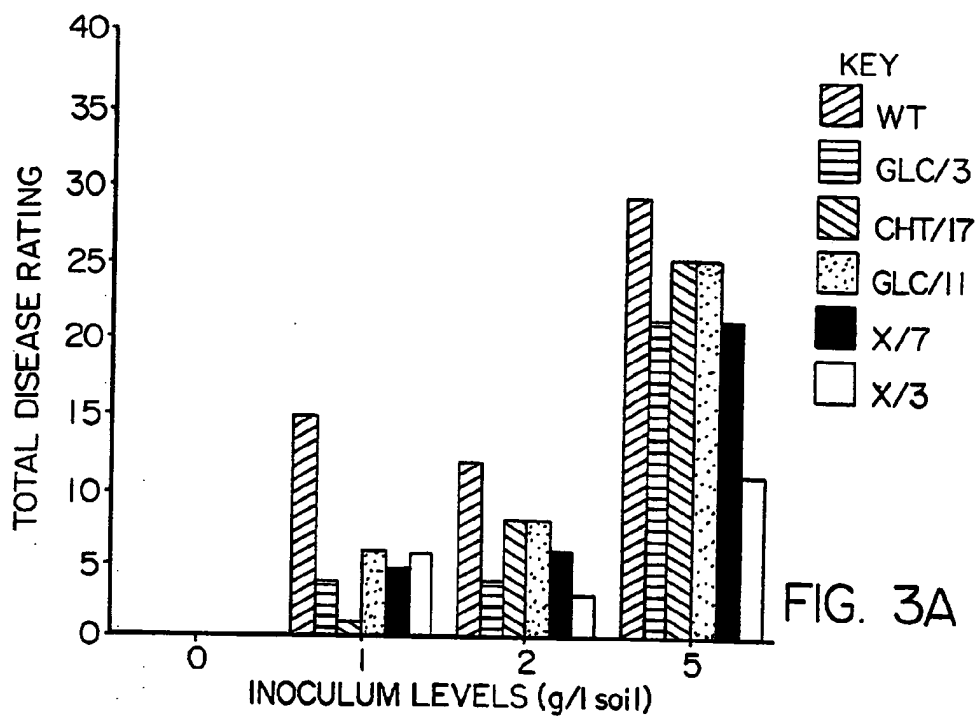
32. A method to increase disease resistance of a transgenic plant containing a first heterologous plant-defense-associated transgene, said method comprising:

introducing a second transgene
5 encoding a second plant-defense-associated
protein into the genome of said plant, wherein
said second transgene is different from said
first heterologous plant-defense-associated
transgene.

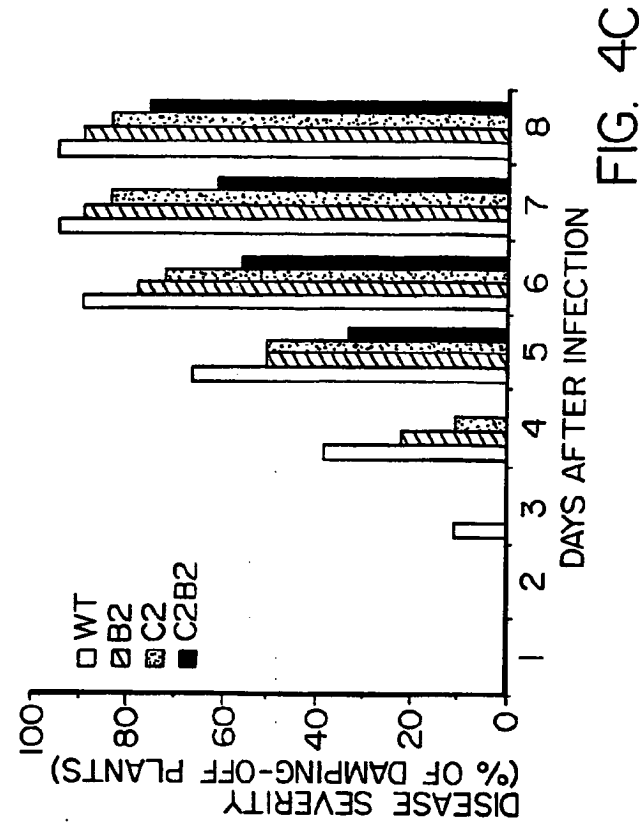
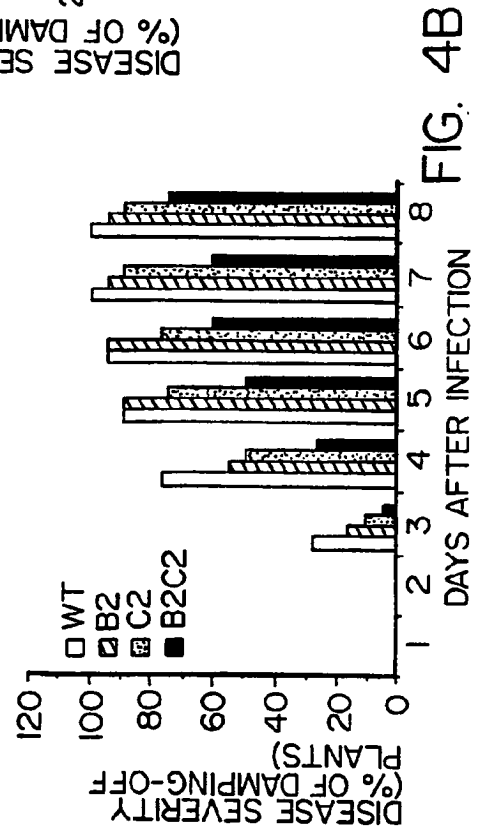
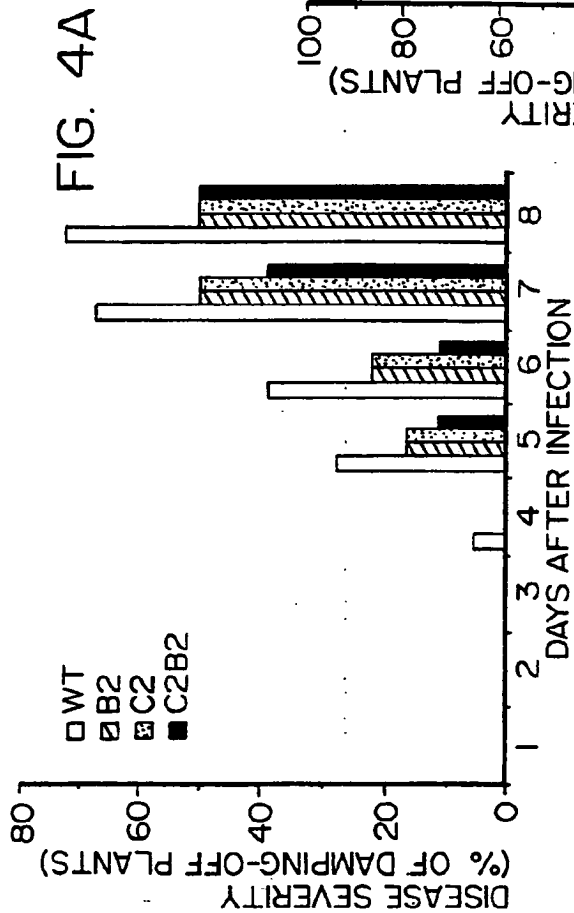
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2 / 3



3 / 3



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/07815

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A01H 1/04; C07H 21/04; C12N 5/00, 9/24, 9/42, 15/00

US CL : 435/172.3, 200, 209, 240.4, 320.1; 536/23.2, 23.6; 800/205

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 200, 209, 240.4, 320.1; 536/23.2, 23.6; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GENBANK, EMBL search terms: SEQ ID NOS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	EP, A, 0,440,304 (CORNELISSEN ET AL) 07 AUGUST 1991, see page 17, lines 1-47, page 18, lines 45-58.	1-7,9,17-19 ----- 8,10-16,20-32
X --- Y	SCIENCE, Volume 254, issued 22 November 1991, K. Broglie et al, "Transgenic Plants with Enhanced Resistance to the Fungal Pathogen <i>Rhizoctonia solani</i> ", pages 1194-1197, see pages 1195-1196.	1 - 5 , 7 , 11 , 12,17-19, 22-24, 28-31 ----- 8,10,13-16,20- 21, 25-27,32



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 SEPTEMBER 1994

Date of mailing of the international search report

24 OCT 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07815

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PLANT SCIENCE, Volume 76, issued 1991, Y. Nishizawa et al, "Rice Chitinase Gene: cDNA Cloning and Stress-Induced Expression", pages 211-218, see page 214.	8,20-21,25
Y	PLANT MOLECULAR BIOLOGY, Volume 16, No. 1, issued January 1991, B. V. Edington et al, "cDNA Cloning and Characterization of a Putative 1,3-Beta-D-Glucanase Transcript Induced by a Fungal Elicitor in Bean Cell Suspension Cultures", pages 81-94, see page 86.	10,20-21, 27
Y	NATURE, Volume 338, issued 16 March 1989, K. Shimamoto et al, "Fertile Transgenic Rice Plants Regenerated from Transformed Protoplasts", pages 274-276, see page 274.	14-16